SYNOPSIS

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Synopsis of research work conducted by **Mukta Deobagkar-Lele** (SR No. 0110-110-007-05584) towards completion of PhD degree under the supervision of **Prof. Dipankar Nandi** at the Department of Biochemistry, Indian Institute of Science, Bangalore, India, 560012.

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Studies on the mechanisms involved in thymic atrophy during *Salmonella enterica* serovar Typhimurium infection

T lymphocytes are an essential component of the adaptive immune response and are highly versatile in function. Each T cell has a unique T cell receptor that can recognize an antigenic peptide in the context of the major histocompatibility complex (MHC) encoded molecules, thus offering a high degree of specificity to the immune response. T cells play a central role in the development of an effective host immune response and the quantitative and qualitative regulation of the T cell response is critical. T cells develop in the thymus, an important primary immune organ, where immature thymocytes undergo differentiation and maturation. Through the process of thymic differentiation, immature cluster of differentiation (CD)4^-CD8^- thymocytes progress to a $CD4^+CD8^+$ stage and are subjected to positive and negative selection to give rise to MHC restricted, single positive $CD4^+$ or $CD8^+$ naive T cells that emigrate from the thymus and populate the peripheral lymphocyte pool.

Thymic atrophy is well known to occur naturally during the process of aging with thymocyte depletion and reduced thymic output. Along with age associated changes leading to atrophy, the thymus is exquisitely sensitive to starvation and several stresses. In addition, thymic atrophy is a characteristic feature during several viral, bacterial and parasitic infections. Egress of immature thymocytes, loss of thymic populations due to sensitivity to glucocorticoids and cytokine modulation, etc. have been variously proposed to be involved in this process. However there is limited understanding on the numerous mechanisms involved and the crosstalk between these diverse pathways.

In this study, a model for thymic atrophy during acute *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) infection was developed. *S. typhimurium* is a Gram negative bacterium that resides and grows in intracellular compartments within host cells. It causes gastroenteritis in humans but leads to typhoid like disease in mice, similar to that caused by *S. typhi* in humans. Initially, it was established that acute infection of C57BL/6 mice with 10^8 CFU *S. typhimurium*, via the oral, i.e. the physiological, route of infection leads to extensive depletion (8-10 fold) of thymocytes in an infection-dependent manner. Infected mice had higher CFU burden in the Peyer's patches, spleen, liver, and mesenteric lymph node (MLN) as compared to the thymus. The thymic atrophy was dependent upon the infection caused by live *S. typhimurium* since oral feeding of mice even with higher doses (10^{10} CFU) of heat-killed bacteria did not lead to thymic atrophy. The susceptible populations in the thymus were identified by staining for expression of CD4 and CD8 on cell surface using specific monoclonal antibodies tagged to fluorophores, e.g. Fluorescein isothiocyanate (FITC) and phycoerythrin (PE), respectively. The double labelled samples were analyzed by flow cytometry. Interestingly, significant death of $CD4^+CD8^+$, the major population of thymocytes, but not single positive thymocytes or peripheral lymphocytes (MLN and spleen cells), was observed at later stages during infection.

To gain greater understanding of the processes involved, the mechanisms leading to thymic atrophy were investigated. To this purpose, small molecule inhibitors and mice lacking key molecules important for the immune response were utilized. Also, various assays to assess death of thymocytes, including analysis of death markers such as Annexin V based detection of membrane flipping and caspase activation were performed.

I. The extrinsic death pathway involving Fas/FasL interactions is a major death pathway. Therefore, the expression and functional role of the components of the pathway in this model of thymocyte death was investigated. It was observed that thymocytes from infected mice expressed more Fas and Fas ligand (FasL) on their surface than cells from uninfected mice. To address the role of the death receptor, Fas, infection studies were performed with *lpr* mice that lack functional Fas expression. The

depletion of $CD4^+CD8^+$ thymocytes in *lpr* mice was comparable to that in C57BL/6 mice indicating that it was independent of the Fas pathway. However, extensive loss of mitochondrial membrane potential was observed upon analysis with mitochondrial potential specific dyes MitoTracker Red and $DiOC_6$. Most likely, the intrinsic death pathway involving mitochondrial depolarization is involved in this model of thymic atrophy.

II. Since thymocytes are known to be sensitive to glucocorticoids both *in vitro* and *in vivo*, the involvement of the same in this model of thymic atrophy was assessed. The amounts of cortisol, a glucocorticoid, as detected by ELISA, were elevated during infection. To investigate the functional implication of the increase in cortisol, studies were performed using RU486, a glucocorticoid receptor antagonist. RU486 did not modulate cortisol amounts and treatment of mice with RU486 did not affect CFU burden or survival of mice. However there was a moderate rescue in the number of viable $CD4^+CD8^+$ thymocytes, with only a 3-4 fold drop as compared to the 8-10 fold drop in vehicle treated infected mice.

III. As glucocorticoids appeared to play a partial role in this model, it was reasonable to assume that other pathways were also involved in the thymic atrophy. The quantitative and qualitative modulation of the cytokine milieu has a profound effect upon the thymus. In fact, inflammatory cytokines, Tnfα and Ifnγ, increased upon infection. In order to study the role of Ifnγ mediated inflammatory responses in this model, infection studies with $Ifn\gamma^{-/-}$ mice were performed. $Ifn\gamma^{-/-}$ mice had higher CFU and lower survival; however the drop in thymocyte numbers was 3-4 fold as compared to the 8-10 fold drop in the infected C57BL/6 mice, again indicating a partial involvement of the Ifnγ mediated pathways.

In order to study the interactions, if any, between the two pathways mentioned above, corticosteroid signaling was blocked in the $Ifn\gamma^{-/-}$ mice with RU486. Upon infection, the number of $CD4^+CD8^+$ thymocytes was significantly higher in $Ifn\gamma^{-/-}$ mice treated with RU486 (~1.5 fold drop in viable thymocyte numbers) along with lower caspase 3 activity and mitochondrial damage. Importantly, cortisol amounts in infected $Ifn\gamma^{-/-}$ mice

were comparable to those in infected C57BL/6 mice and the administration of RU486 did not modulate Tnfα and Ifnγ cytokine amounts in sera. Thus, the glucocorticoid and Ifnγ mediated pathways are parallel but synergize in an additive manner to induce death of $CD4^+CD8^+$ thymocytes during *S. typhimurium* infection.

IV. Although thymic atrophy is known to occur, a detailed characterization of cell surface changes in thymocyte populations has not been performed. To investigate this aspect, thymocytes and MLN cells from uninfected and infected animals were stained for cell surface expression of CD3, CD4, CD5, CD8, CD24, CD25, CD44, CD69, MHC I and MHC II. This analysis was initially performed by studying the changes in expression of these molecules within the total thymocyte and MLN populations. Although there was no change in the expression of CD25 and MHC II in the total thymocyte population upon infection, CD24 expression reduced, whereas, the expression of CD3, CD5, CD44, CD69 and MHC I increased. Notably, changes in the frequency of expression of CD3, CD69 and MHC I were observed before the development of extensive thymic atrophy. The depletion of majority of the $CD4^+CD8^+$ thymocytes enriches the mature $CD4^+$ or $CD8^+$ thymocyte population This was corroborated with the observation that, upon *in vitro* stimulation with PMA and Ionomycin (pharmacological agents used to activate T cells) the residual thymocytes from infected mice produced more IL2 compared to thymocytes from uninfected mice.

Subsequently, cells were stained with anti-CD4-FITC, anti-CD8-PE and a third biotinylated antibody, which was detected by a streptavidin-APC conjugate, against one of the remaining six markers. This three colour analysis made it possible to determine the changes in the expression of the third marker in each of the $CD4^-CD8^-$, $CD4^+CD8^+$, $CD4^+$ and $CD8^+$ populations upon infection. Distinct differences were observed in the phenotypes of uninfected and infected $CD4^+CD8^+$ thymocytes and the latter were $CD3^{high}$, $CD5^{high}$, $CD24^{low}$, $CD69^{high}$ and MHC I^{high} indicating that the surviving population had a possibly more mature phenotype. Also, the changes in the phenotypes of the thymocyte populations were dependent upon the extent of thymic atrophy as indicated by time course and CFU studies with C57BL/6 and BALB/c mice respectively. Finally, the roles of glucocorticoids, Ifnγ and Nos2 in modulation of

expression of these markers during infection were addressed. Interestingly, the expression of CD3, CD24 and MHC class I significantly correlated with increase in the number of surviving thymocytes upon inhibition of glucocorticoids signaling and in *Ifnγ*$^{-/-}$ mice. The implications of these changes in the thymocyte surface phenotype during thymic atrophy are discussed.

V. Finally, the roles of downstream signalling molecules in *S. typhimurium* induced thymic atrophy were studied. Although the MAP kinase family members, Erk, Jnk and p38 have been implicated to play a role in the positive and/or negative selection of thymocytes during development, their role in infection induced thymocyte depletion has not been studied. Interestingly, the amounts of Jnk and pJnk, but not p38, increased in thymocytes upon infection. Importantly, pJnk amounts increased predominantly in CD3$^{-/low}$ thymocytes during infection. Furthermore, inhibition of Jnk signalling, using a specific inhibitor SP600125, lead to an increase in survival of CD4^{+}CD8^{+} thymocytes during infection due to multiple reasons: lowering of cortisol, Tnfα and Ifnγ amounts, and better maintenance of thymic architecture. Thus, inhibition of Jnk mediated signaling protected CD4^{+}CD8^{+} and CD3$^{-/low}$ thymocytes from death during *S. typhimurium* infection.

Overall, the main conclusions of this study are as follows: First, extensive analysis of the surface phenotype of cells during thymic atrophy throws light on the sensitive and resistant thymocyte populations, thus offering a potential predictive marker profile. Second, glucocorticoids, Ifnγ and, importantly, Jnk mediated signaling play functional roles in the death of immature CD4^{+}CD8^{+} thymocytes during *S. typhimurium* infection. The mechanistic details uncovered in this study may be important in designing effective strategies for reducing thymic atrophy during other infections. In fact, enhancement of thymic output may lead to greater numbers and diversity of thymic T cell emigrants in the periphery which is likely to enhance host responses during infections.